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RESEARCH ARTICLE

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A Comparison of Bacteriological Culture, Serology, and Quantitative PCR for Detecting Brucellosis in Ewes with a History of Abortion

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ABSTRACT

The zoonotic disease brucellosis is a serious public health and livestock industry concern. In the present study, we used bacteriological culture, RBT, and qPCR to determine the prevalence of brucellosis in the serum and milk samples of sheep with a history of abortion. Serum and milk samples were obtained from 100 sheep aged 3-5 years. In order to determine the prevalence of brucellosis, a modified RBT was performed on serum samples, Brucella was isolated from milk by bacteriological culture, and qPCR was applied to detect bacterial DNA in milk. The prevalence of brucellosis using modified RBT, bacteriological culture, and qPCR was 32%, 42%, and 44%, respectively. By considering qPCR as the standard, modified RBT showed a sensitivity of 95%, a specificity of 100%, an accuracy of 98%, a PV+ of 100%, and a PV- of 97%. The sensitivity, specificity, accuracy, PV+, and PV- for bacteriological culture were 77%, 100%, 90%, 100%, and 85%, respectively. The agreement between qPCR and modified RBT was 0.959 (95% CI: 0.896-1), between qPCR and bacteriological culture was 0.792 (95% CI: 0.667-0.897), and between modified RBT and bacteriological culture was 0.831 (95% CI: 0.709-0.38). Based on the results, bacterial isolation from sheep milk is not recommended except in specific cases due to its low sensitivity, as well as its time-consuming and hazardous nature. However, the modified RBT can be used as a routine method because of its cost-effectiveness, higher sensitivity, and higher accuracy compared to bacterial isolation. Moreover, qPCR is recommended as the gold standard test for detecting brucellosis in sheep milk, especially in those with a history of abortion.

Keywords

Brucellosis, Modified Rose Bengal, qPCR, Sheep

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Abbreviations

RBT: Rose Bengal test
qPCR: quantitative PCR
CI: Confidence Interval
PV+: Positive Predictive Value

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Introduction

The *Brucella* genus is a non-motile, gram-negative, and intracellular *coccobacilli* bacteria that causes the zoonotic disease brucellosis [1-3]. Human Malta fever caused by *Brucella*, with more than 500,000 cases annually, is the most common contagious disease between humans and farmed species worldwide [4]. The genus Brucella, with 12 main species, can cause disease in several animal breeds, leading to economic loss. For example, abortion, stillbirth, and reproductive disorders are common clinical manifestations of brucellosis in sheep [5, 6]. Human infections are mainly caused by B. *melitensis*, B. *abortus,* B. *suis*, and B. *canis*, of which B. *melitensis,* with three biovars, is the most contagious [7-9]. *Brucella* infects humans by direct contact with contaminated tissues (e.g., placenta, fetus, and uterine secretion) or by consuming unpasteurized dairy products [10, 11]. Contrary to the cow milk used in industrial dairy production, ewe milk is routinely used for producing raw milk products, increasing the risk of contracting Malta fever [12]. In addition, B. melitensis, as the major causative agent of *brucellosis* in ewes, displays higher pathogenicity in humans than B. *abortus* due to its 10,000 times lower infectious dose [13]. As mentioned, *brucellosis* significantly impacts the livestock industry and public health. The control strategies are based on prevention and eradication. Sheep infected by *Brucella* are considered reservoirs in herds. In order to lessen the risk of disease and subsequent economic losses, the infected sheep must be identified and removed from the herd by the fastest, most cost-effective, and least hazardous method. Indeed, the detection of *Brucella* is the fundamental step in any control program. To achieve this goal, laboratory diagnosis could be performed in three diverse areas: 1) direct detection of living bacteria using culture media, 2) indirect diagnosis by serological methods, and 3) rapid diagnosis by molecular assays based on PCR [14].

Bacterial isolation is the most accurate method for brucellosis detection. However, the chronic stage of the disease is challenged by several limitations, including a long incubation period and low sensitivity. In addition, appropriate safety precautions need to be implemented for exposed laboratories and workers due to the hazardous nature of the Brucella organism classified as a class III pathogen [15]. Although serological methods are recommended for detecting brucellosis, they can have false positive and/or false negative results. Indeed, they are either too sensitive causing false positives, or too specific causing false negatives [16]. Moreover, the presence of antibodies in a serum sample does not always indicate an active case of brucellosis. Sustained immune responses that form after vaccination are an example of antibody formation in the absence of brucellosis [17]. Furthermore, in serological tests several gram-negative bacteria, especially *Salmonella* group N (O: 30), Escherichia coli O157:H7, Yersinia enterocolitica O:9, and Vibrio cholerae O1, can induce antibodies with cross-reactivity and cause false-positive results for brucellosis [18]. Therefore, employing two serological tests simultaneously to decrease the number of false positive and false negative results is highly recommended. According to the available protocols in Iran, RBT is applied for primary screening. Next, SAT and 2-ME confirm positive RBT samples. Despite the limited and conflicting information about RBT [19, 20], this test has been internationally approved for monitoring brucellosis in small ruminants [21]. Rose Bengal can be used as a rapid test for monitoring, but more specific tests are needed to confirm RBT results. SAT is routinely used for confirmation, and titers above a certain threshold are considered active brucellosis. Moreover, 2-ME, combined with SAT, differentiates between the agglutination of IgG and IgM-specific antibodies [22]. Because of the problems raised by the bacteriological culture and immunological methods, developing new diagnostic examinations for directly detecting Brucella species in milk has been increasingly under investigation. Recently, qPCR, as a well-established method, has been widely used to detect unculturable or slow-growth bacteria in microbial communities. The number of investigations on *Brucella* detection from ewe milk by qPCR is relatively limited. Consequently, evaluation of the efficiency of this method for detecting Brucella in ewe milk is not applicable. However, it seems that a molecular detection method, such as qPCR, which targets the specific region of Brucella with high sensitivity, could be an appropriate approach for the rapid and safe diagnosis of *Brucella* with the lowest rate of false negative and false positive results. We conducted a real-time PCR assay based on designing an alternative pair of primers to detect *Brucella. spp*. The present study aimed to: 1) determine the prevalence of brucellosis in ewes with a history of abortion by bacterial culture and qPCR on milk samples and serological methods (Rose Bengal, Wright, 2-ME) on serum samples, 2) compare the efficiency of three diagnostic methods (molecular, serological, and bacteriological) for identifying the infected ewes, and 3)

Abbreviations-Cont'd

PV-: Negative Predictive Value
SAT: Serum agglutination test
2-ME: 2-mercaptoethanol
LR: Likelihood ratios
MRT: Milk ring test
ELISA: Enzyme-linked
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

detect B*rucella* species circulating in the ewe population by Bruce-ladder multiplex PCR assay.

Result

To ensure the efficiency of DNA extraction from milk, all samples were evaluated for the integrity of GAPDH (housekeeping gene) in sheep. The 467bp GAPDH amplicon detected on a 1% agarose gel (Figure 1) showed an appropriate DNA extraction efficiency.

Comparison of Serological Tests and Culture with qPCR

Out of 100 milk specimens, Brucella spp. were isolated from 34 samples (34%), demonstrating the phenotypic and biochemical characteristics of typical Brucella species, including small and smooth colonies, non-hemolytic small gram-negative coccobacilli, catalase positivity, oxidase positivity, and urease positivity. All the isolates grew well in both aerobic and 8%-10% CO_2 atmospheres at 37°C, 4-8 days after incubation. Cultures that did not show any sign of growth until day eight did not grow until the end of 16 days in either atmosphere. Along with the phenotypic assays, the isolated bacteria were confirmed by PCR using genus-specific primers (Figure 2). Moreover, the species of *Brucella* strains were defined by the Bruce-ladder multiplex-PCR as well. Forty-two serum samples (42%) were diagnosed as positive by all three serological tests. Furthermore, all the positive cultures showed positive results in the serological tests. However, eight samples with positive serological results did not show any growth in culture. Using qPCR, the genomic elements of Brucella spp. were detected in 44 milk samples (44%), 42 of which were serologically positive. All the serological- and culture-positive samples were also positive in qPCR (Tables 1 and 2).

The sensitivity, specificity, PV+, and PV- of serological tests and cultures were calculated based on the qPCR results. The sensitivity, specificity, PV+, and PV- of serological tests compared to qPCR were 95%, 100%, 100%, and 97%, respectively. The mentioned parameters for microbial culture compared to qPCR were 77%, 100%, 100%, and 85%, respectively.

Using the Kappa test, all three methods were evaluated for inter-rater reliability. The agreement between qPCR and modified RBT was 0.959 (95% CI: 0.896-1), between qPCR and culture was 0.792

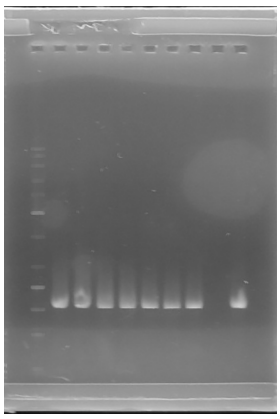

Figure 1.
PCR product of GAPDH gene. Lane 1: 100-bp DNA size marker (100-1500 bp); Lane 2-8: GAPDH gene; Lane 9: Negative control; Lane 10: Positive control

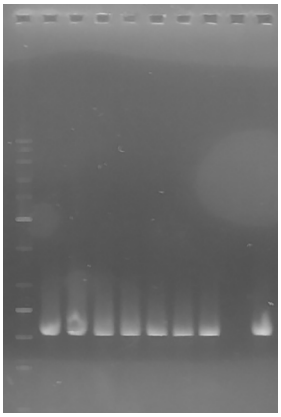

Figure 2.
PCR product of Brucella spp. Lane 1: 50-bp DNA size marker (50-1k bp); Lane 2-8: Brucella spp.; Lane 9: Negative control; Lane 10: Positive control

Table 1.
Prevalence of brucellosis in sheep with a history of abortion based on the positive results in each diagnostic test, n (%)

Sample (n)	Microbial culture	RBT	qPCR
100	34 (34%)	42 (42%)	44 (44%)

Table 2.
Data obtained from Rose Bengal Test, microbial culture, and qPCR, including true positive (a), true negative (d), false positive (b), and false negative (c) results

qPCR	Brucella (Modified RBT)		Total	Brucella (Microbial culture)	
	Negative	Positive		Negative	Positive
Positive	(a) = 42	(b) = 2	(a+b) = 44	(a) = 34	(b) = 10
Negative	(c) = 0	(d) = 56	(c+d) = 56	(c) = 0	(d) = 56
Total	(a+c) = 42	(b+d) = 58	n= 100	(a+c) = 34	(b+d) = 66

(95% CI: 0.667-0.897), and between modified RBT and culture was 0.831 (95% CI: 0.38-0.709). The positive and negative LR of the diagnostic tests used in this study were also evaluated (Table 3). A positive Rose Bengal or/and culture result is ∞ (infinity) times more likely to originate from an infected animal than from a healthy animal. Only 0.05 times as many animals with brucellosis as animals without the disease will provide a negative Rose Bengal result. An infected animal is 0.23 times more likely to have a negative culture result than a healthy animal.

Bruce-ladder Multiplex PCR

Regarding the capability of Bruce-Ladder multiplex PCR in identifying the Brucella species which are isolated in pure cultures, the test was performed on 32 extracted DNA samples of Brucella bacteria isolated from pure bacterial cultures. The patterns of produced fragments on the 1.5% agarose gel patterns were evaluated by Yoldi et al. All the isolates (Figure 3) were identified as B. melitensis (six amplicons with sizes of 152-bp, 450-bp, 587-bp, 794-bp, 1071-bp, and 1682-bp were multiplied).

Table 3.
Statistical parameters for modified RBT and microbial culture compared to qPCR for the diagnosis of brucellosis (95% CI

Statistic parameter based on qPCR	Value	
	Modified RBT	Culture
Sensitivity	95%	77%
Specificity	100%	100%
Positive Likelihood Ratio	∞	∞
Negative Likelihood Ratio	0/05	0/23
Positive Predictive Value	100%	100%
Negative Predictive Value	97%	85%
Accuracy	98%	90%
Kappa	95% CI: 0.896-1)0.959	95% CI: 0.709-0.38)0.831)
	(95% CI: 0.667-0.897)0.792	

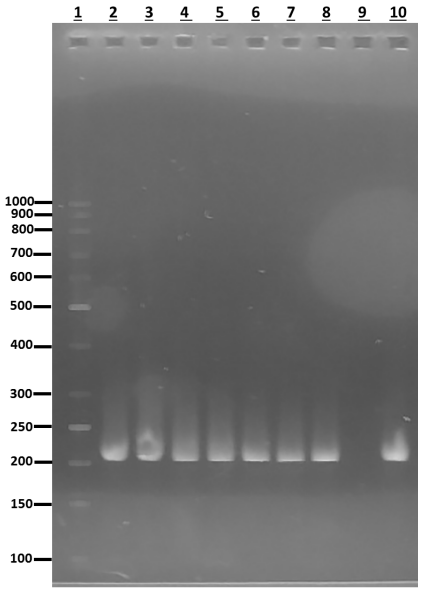

Figure 3.
Differentiation of B. abortus, B. melitensis, RB51, and Rev.1 vaccine strains by Bruce-ladder multiplex PCR. Lane 1: 100-bp Plus DNA size marker(100-3k bp); Lane 2: B. abortus; Lane 3: B. melitensis; Lane 4: B. abortus RB51 vaccine strain; Lane 5: B. melitensis Rev.1 vaccine strain

Discussion

Brucellosis due to B. *melitensis* is still a major problem for public health and also for sheep herds in several parts of the world, especially in the Middle East and the Mediterranean region. Most human cases of *brucellosis* around the world are infected with this species of Brucella. B. melitensis, the most important zoonotic pathogen between humans and animals, primarily infects sheep as its preferred host and transmits to humans mostly by consuming the milk and dairy products of sheep and goats which are unpasteurized, especially in endemic areas [8, 23-29]. Some clinical symptoms of brucellosis in sheep include abortion, stillbirth, retained placenta, weak lambs, and infertility which cause significant economic loss to the livestock industry [30]. In areas with a high prevalence of *brucellosis* (more than 5%), B. *melitensis* Rev. 1 strain vaccine is recommended on a large scale or/and for maiden ewes [28]. In the current study, sheep milk samples were directly subjected to molecular investigation for *Brucella spp*. DNA extraction was completed according to

Pokorska *et al.* [31], which showed the advantages of low cost, short time, and less volume of milk compared to many other methods. Studies on the prevalence of brucellosis in sheep have been conducted in Iran and other parts of the world using different methods and conditions of sheep (with a history of abortion or not). In the current research, the prevalence of brucellosis in ewes with a history of abortion was determined by three assays. In the milk culture, 34% of sheep were *Brucella*-positive, while serological methods and qPCR on milk samples determined the prevalence of *brucellosis* as 42% and 44%, respectively. As Al-Talafhah AH *et al.* [32] reported, monitoring the herd status in northern Jordan by RBT showed that 61% of all herds and 14% of sheep in each herd were positive for brucellosis. In another study conducted by Samadi A. et al. [33], 86 out of 188 (45.7%) samples of sheep with a history of abortion were positive for brucellosis using PCR. Zhang H *et al.* [34] reported that in the fetal tissues and milk of 120 sheep and cows, PCR for brucellosis was positive for 34 samples (28%). Therefore, there are some similarities and differences between the findings of this study and others. Differences in the prevalence of brucellosis can be due to variations in sample types or methods applied in each investigation. In a section of the study conducted by Hamadi et al. [17], blood and milk samples of 21 sheep were evaluated for brucellosis using RBT, culture, and PCR. Twenty samples were seropositive for RBT. *Brucella spp*. were isolated from 12 milk samples, while PCR detected Brucella spp. in ten milk samples. Eleven PCR-negative samples were positive in RBT, while a single Rose Bengal-negative sample was positive with PCR. In a study by Gupta et al. [6], out of 54 goat samples with a history of abortion, 32 serum samples were positive for SAT. Brucella genomic fragments were amplified in 48 milk samples, including 32 serum-positive specimens. It was found that PCR, as a controlled experiment, had a specificity of 100% and a sensitivity of 90%. Ilhan et al. [2] indicated that by examining the milk samples of sheep with a history of abortion, 8, 24, and 28 samples tested positive in culture, PCR, and MRT, respectively. Comparing MRT and PCR, 22 positive and 72 negative samples were common in both tests, and a coincidence of 96% was achieved. For PCR, the specificity and sensitivity were estimated at 100% and 81.3%, while for MRT, these indices were 75% and 75%, respectively. Altun *et al.* [35] evaluated 65 sheep milk samples for antibodies against *Brucella* with indirect ELISA and *Brucella* DNA with qPCR. According to their findings, 6.1% of the samples tested positive in both examinations. Lindahl et al. [16] examined blood samples with indirect ELISA and milk samples with qPCR from 570 non-vaccinated cattle. All serum-positive samples were also positive with qPCR, while 8.3% of seronegative cows tested positive for *Brucella spp.* DNA in their milk. In a study performed by Sabrina *et al.* [36], milk samples were obtained from 65 seronegative cows and tested for genomic fragments of Brucella with qPCR. Results revealed that 3.08% of cows tested positive for Brucella contamination. Zakaria [37] conducted research using 230 blood samples to establish the prevalence of brucellosis by RBT, modified in-house ELISA, and qPCR. The sensitivity and specificity of two serological tests were also calculated using qPCR as a standard. The overall prevalence of brucellosis was estimated at 53.9%, 75.2%, and 79.1% for ELISA, RBT, and qPCR, respectively. The sensitivity of RBT was 79.12%, and that of ELISA was 55.49%. In the present study, qPCR identified more positive samples (44 samples) than the culture method (34 samples) which indicates the higher sensitivity of qPCR than microbial culture for detecting brucellosis. Similar results were indicated in studies [37-39] comparing culture and conventional PCR on cow milk, which can be generalized to this study based on the higher sensitivity of qPCR than the conventional PCR. These results could be linked to the fact that in molecular methods, by targeting the genome of *Brucella*, both live and dead organisms could be detected, while in the culture method, only live organisms could be recognized by growing on a culture medium. Since a small number of *Brucella* organisms can cause the disease, the molecular approach seems more suitable than the culture method for identifying brucellosis in infected animals for the control and eradication purposes.

No serological test has been specially defined for B. melitensis infection in sheep. It is commonly assumed that the serological tests used for identifying B. *abortus* in cows are sufficient to diagnose B. melitensis infection in sheep and other small ruminants, such as RBT which is widely used to diagnose *brucellosis* in sheep while it is mainly designed for B. *abortus*. Standardizing the antigens is a major challenge that affects the sensitivity of RBT. The antigen standardization conditions that seem suitable for detecting B. *abortus* in cows are insufficient for B. *melitensis* diagnosis in sheep [20, 40]. Moreover, RBT has specific limitations, including anti-complementary activity, the prozone effect that requires heat-inactivated serum [41], and low sensitivity confirmed in culture-proven cases [15, 42]. The Rose Bengal serology test used in this study demonstrated negative results for two sheep, while qPCR detected Brucella genomic fragments in the milk of these sheep. The results were similar to those reported by Leal-Klevezas et al. [38]. Despite it is recommended [40] that increasing the volume of serum can enhance the sensitivity of the RBT, the result of the present study showed that this Modified RBT can still have false negatives compare to the presented qPCR. This finding alarms and confirms that the sensitivity of RBT when testing blood samples of sheep requires improvement. However, modifying the antigen used in RBT by reducing the pH or cell concentration of the antigen may enhance the RBT sensitivity to an acceptable level when using sheep serum.

The qPCR protocol proposed in our study demonstrated advantages over the conventional microbial culture method, including higher speed and greater sensitivity. Moreover, there is no requirement for live *Brucella* organisms in this method which reduces the chance of infection transmission to laboratory staff and increases safety. Finally, it is recommended to use qPCR to diagnose or confirm the presence of B. melitensis in sheep milk as a stand-alone method or in combination with other techniques as a part of control and prevention programs. Although estimating the prevalence of brucellosis was not the main objective of our study, the results revealed that despite vaccination and other control measures over the years, clinical brucellosis still exists in sheep in various parts of the country and is one of the main causes of both sheep abortion and human brucellosis. This study was conducted on a small population of sheep with a history of abortion. Consequently, further extensive research at the national level is required to target the whole population of traditional and nomadic herds [26] using qPCR alongside other diagnostic methods as a sensitive, accurate, rapid, and easy technique. That can prevent the remaining infected sheep from being a false negative source of contamination in the herd.

Conclusion

One of the main measures of the control and prevention program for brucellosis is identifying infected animals. Screening is the first and most important step in the test-and-slaughter strategies. The discrepancy between the serological methods and qPCR highlights the need for additional diagnostic strategies to detect serologically false negative animals in screening, control, and eradication programs for brucellosis. However, in countries with limited resources, test-and-slaughter cannot be implemented. Therefore, identifying infected animals in herds allows farmers to take appropriate protective measures to reduce the spread of the disease.

Materials and Methods

Sampling

A total of 200 milk (n = 100) and blood (n = 100) samples were collected from ewes of different flocks with a history of abortion, aged 3-5 years, vaccinated with Rev.1 vaccine at the age of 6 months, which had not received any antibiotic or corticosteroids for at least one month before sampling. Following disinfection with 70% alcohol, blood specimens were taken from the jugular veins using 5 mL sterile syringes and were collected in tubes without anticoagulant. Before collecting milk samples, each teat was washed with warm water and wiped with a disposable towel. Initially, the first squirts of milk were disposed of. Then, about 10 mL of milk was collected from every teat in a sterile 50 mL Falcon tube. To prevent cross-contamination, the gloves were changed after each sampling. After taking the specimens under hygienic conditions, they were kept on ice and transferred to the laboratory within a maximum of 3 hours. The milk in the falcon tubes was divided into two sterile 15 mL tubes under laboratory conditions, one of which was used right away for microbial culture, while the second tube was stored at a temperature of -80°C for conducting molecular experiments in the future. It should be noted that all the manipulations of the samples and cultures in the laboratory were performed in a class II biological safety cabinet and national and international guidelines for dealing with Brucella-contami-

nated materials were followed.

Serological Test

Serological tests are a part of control and eradication programs for the detection of B. melitensis infection in ruminants.

Rose Bengal Test

To reduce false negative results, modified RBT, introduced by Blasco et al. [40], was used to increase sensitivity without affecting specificity [40, 43]. Briefly, 75 µL of the sera obtained from the studied ewes were mixed with 25 µL of Brucella antigen (Razi Vaccine & Serum Research Institute, Iran) at room temperature on a flat white ceramic plate and gently shaken for 4 minutes. Any agglutination that appeared during this time was recorded as a positive reaction.

Serum Agglutination Test and 2-MercaptoEthanol Test

The Wright and 2-ME tests were applied to confirm the positive results of modified RBT. For SAT, serum samples were prepared using a solution of sodium phenol chloride with a dilution ratio of 1:80, mixed with an equal volume of Brucella antigen (Wright Tube Kit®, Pasteur Institute, Iran) resulting in a 2-fold dilution. After incubating samples for 24 hours at 37°C, they were examined for agglutinated particles, and serum titers of 1:80 or higher were considered positive. The 2-ME test was performed for SAT-positive serum samples, with a 1:4 ratio of serum and the 2-ME solution mixed and incubated at 37°C for an hour. Next, a solution of sodium phenol chloride with a dilution of 1:80 was added, resulting in a 2-fold dilution of the reactions. After incubating for 24 hours at 37°C and resting for 1 hour at room temperature, the serum specimens were examined. A positive result was reported for the 2-ME test when the serum titers were 1:40 or greater.

Microbial Culture and Bacterial Isolation

Samples and Brucella strains were cultured in the CITA selective culture medium described by De Miguel et al. [44], which was also recommended by the World Organisation for Animal Health (OIE) for the isolation of *brucella* isolates, especially smooth Brucella species, such as B. *melitensis* and B. *abortus*. Briefly, the CITA selective medium consists of blood base agar plates containing 5% sterile sheep serum and is supplemented with antimicrobial agents as follows: antifungal agents amphotericin B (4 mg/L) and nystatin (100 000 IU/L) (Solarbio Science & Technology Co., Beijing, China), as well as antibiotics vancomycin (20 mg/L), colistin (7.5 mg/L), and nitrofurantoin (10 mg/L) (Solarbio Science & Technology Co., Beijing, China). Milk specimens were centrifuged at 3000 ×g for 15 min at 4°C. Afterwards, loopfuls of both cream and sediment were used for simultaneous inoculation onto two CITA plates. The plates were then incubated in two different atmospheres: aerobic and with 8%-10% carbon dioxide (Microbiology Anaerocult c®, Merck, Darmstadt, Germany) at 37°C for up to 16 days. The plates were evaluated for bacterial growth every 3 days starting from day 4, and if no growth was observed after day 16, the culture was reported as a negative result. In the case of bacterial growth observation, a pure culture was prepared for further phenotypic and molecular confirmation. Phenotypic characteristics for confirming *Brucella. spp*, such as colonial morphology, bacterial morphology, gram staining, catalase, oxidase, and urease activity were recorded.

Molecular Tests

In parallel to the microbial culture, the molecular method using the DNA extracted from the isolated strains was applied for genotypic identification using genus-specific primers for genus detection and Multiplex Bruce-ladder PCR for the diagnosis of Brucella species.

DNA Extraction

DNA was extracted from pure cultures using the modified boiling method introduced by Queipo-Ortuño et al. [45]. In summary, the bacteria obtained from pure culture were washed twice with Tris-HCL-EDTA buffer and centrifuged at 15000 ×g for 10 min. Approximately 600 µL of the top layer of the second centrifugation was removed, and the tube with the remaining material was incubated in a water bath at 100°C for 10 min. After keeping it on ice for 10 min, the tube was centrifuged at 15000 ×g for 10 min. The supernatant was separated and placed at -20°C for further use. To perform DNA extraction from milk samples, we followed the method previously described by Pokorska *et al.* [31]. In brief, 10 mL of milk collected during sampling was centrifuged at 7000 ×g for 10 min at 4°C. The liquid layer on the top of the tube along with the fat from the milk was removed, and the remaining pellet at the bottom of the tube with its supernatant liquid was transferred to a sterile 2 mL tube. The mixture then underwent the process of centrifugation at 5000 ×g for 3 min at 4°C, and the liquid layer on top was removed. The pellet was washed with 1 mL of buffer (15 mM Tris-HCl (pH 7.4-7.6), 25 mM NaCl, 5 mM $MgCl_2$, 15 mM Na_2HPO_4, 2.5 mM EDTA, 1% sucrose) by centrifuging at 5000 ×g for 3 min at 4°C, and discarding the supernatant liquid. This step was repeated until the supernatant liquid became clear. Then, 1 mL of lysis buffer (pH 8.8; 6% SDS, 3 mM $MgCl_2$, 15 mM Tris-HCl, 0.5% DMSO, 6% acetone) was added to the pellet obtained from the preceding step and incubated at 65°C in a water bath for about 60-90 min until the pellet was dissolved entirely. Next, the mixture was cooled at room temperature, and 450 µL of precipitating buffer (2.35 M NH_4Cl, 1.15 M NaCl, 38% ethanol pH: 5) was added. After Vortexing and centrifuging at 16000 ×g for 8 min at 10°C, the liquid on the surface was transferred to a new tube, and 600 µL of 100% isopropanol was added. The tube was then centrifuged at 10000 ×g for 8 min, and the remaining liquid on the surface was removed. The DNA pellet obtained was washed twice with 70% ethanol and air-dried. Next, the DNA pellet was dissolved in 100 µL of TE buffer (pH 8.0, 10 mM Tris, 1 mM EDTA). Quality and quantity assessment of DNA extracted from milk was beyond the main objectives of this study. However, To confirm the successful DNA extraction process from milk samples, the primers described by Kadivar et al. [46] were applied to amplify a 467 bp sequence of a housekeeping gene known as the glyceraldehyde-3-phosphate dehydrogenase (NC_056056.1). Calibrated 1% agarose electrophoresis (XM_060411591.1)) and Green Viewer safe stain (0.01 v/v) were used to assess the PCR products. A 100-bp DNA ladder (100-1500 bp) (Cat No.YT8503, Yekta Tajhiz Azma, Tehran, Iran) was used as a DNA marker. The sample was stored at -20°C for further examination if the result was positive.

PCR and Bruce-ladder

The *Brucella spp*. molecular confirmation was conducted on the DNA samples, which were extracted from Brucella genus identified positive bacteriologically using genus-specific primers (Metabion International AG, Planegg, Germany) according to the procedures proposed by Richtzen et al. [47] and calibrated 1% agarose electrophoresis was used with a 50-bp DNA size marker (50-1k bp) (DNA ladder III®, Cat No. S-5092-100, Dena Zist Asia, Mashhad, Iran). The characteristics of the primers applied in the current study are presented in Table 4. Furthermore, considering the capability of the Bruce-ladder multiplex PCR in identifying Brucella species (B. *melitensis*, B. *abortus*, B. *ovis*, B. *melitensis*, B. *canis,* B. *neotomae*, B. *pennipidialis*, and B. *ceti*) and

vaccine strains (B. abortus S19 vaccine strain, B. abortus RB51 vaccine strain and B. *melitensis* Rev.1 vaccine strain), detection of Brucella species was carried out on *Brucella* genus identified positive using genus-specific primers and the Bruce-ladder multiplex PCR as described by García-Yoldi D *et al.* [48]. In summary, using a thermocycler device (Gene Atlas 322®, Astec Co., Fukuoka, Japan) with 20 µL mixture containing 10 µL of Taq 2x Master Mix Red (Ampliqon A/S, Odense, Denmark), 4 µL of a primer mixture (Metabion International AG, Planegg, Germany), 1 µL of template DNA, and 5 µL of UltraPure™ DNase/RNase-free distilled water, PCR was performed. The PCR program included an initial denaturation at 95°C for 7 min, followed by 25 cycles of 35 sec of template denaturation at 95°C, 45 sec of annealing at 64°C, and 180 sec of extension at 72°C, with a final extension at 72 °C for 6 min. PCR products were analyzed by calibrated 1.5% agarose electrophoresis with Green Viewer safe stain (0.01 v/v) and a 100-bp Plus DNA size marker (100-3k bp) (DNA ladder II®, Cat No. S-5091-100, Dena Zist Asia, Mashhad, Iran). Moreover, the B. melitensis Rev.1 vaccine strain, B. abortus RB-51 vaccine strain (used in the vaccination program of the Iranian Veterinary Organization), and B. melitensis strain were used as positive controls.

qPCR Design and Setup

To detect most variants of Brucella, primers were designed for the conserved region of the complete genome sequence of B. *ceti* (NC_022905.1), B. *abortus* (NC_007618.1), B. *melitensis* (NC_003317.1), B. canis (NC_010103.1), B. *microti* (NC_013119.1), B. *neotomae* (NZ_UIGH01000001.1), B. *ovis* (NC_009505.1), and B. *suis* (NC_004310.3) by beacon designer (version 8.10, Premier Biosoft, USA) (Table 4). Using the Basic Local Alignment Search Tool from the GeneBank database and Snapgene software (version 3.2.1, USA), the in silico specificity was examined. The qPCR was conducted using a 10-µL mixture containing 5 µL of Real Q Plus 2x Master Mix Green (Ampliqon A/S, Odense, Denmark), 1 µL of reverse and forward primers (Metabion International AG, Planegg, Germany), 1 µL of template DNA, and 3 µL of UltraPure™ DNase/RNase-free distilled water. Amplification and detection were performed using a real-time device (mic-PCR®, Applied Biomolecular Systems Co., Australia). The activation step was carried out at 95°C for 15 min, and the template was subjected to a total of 35 cycles comprising 30 sec of denaturing at 95°C and 30 sec of annealing at 60°C. After completing the annealing step, melting curve analysis was performed within the temperature range of 65°C-95°C. The baseline and threshold were set using the auto baseline and threshold feature in mic-PCR® Software v2.6.4 (Applied Biomolecular Systems Co., Australia). Before data analysis, the melting curve (Figure 4) was recorded for each reaction, and by examining these curves, the accuracy of the peak related to the desired DNA fragment and the absence of primer dimers was confirmed. Moreover, in all qPCRs in our study, if the cycle threshold (Ct) values were 35 or lower,

Table 4.
Characteristics of the primers used in the PCRs

Primer Pair	Primer Name	Sequence (5'to 3')	Amplicon Size (bp)
GAPDH		F:TGGCAAAGTGGACATCGTTG	467
		R:TGGCGTGGACAGTGGTCATAAGTC	
Genus Brucella		F: TGGCTCGGTTGCCAATATCAA	223
		R: CGCGCTTGCCTTTCAAGGTCTG	
qPCR		F: TCCTCGGTCCAGACATAG	142
		R: GCGATGATTTATTCCGTATCCa	

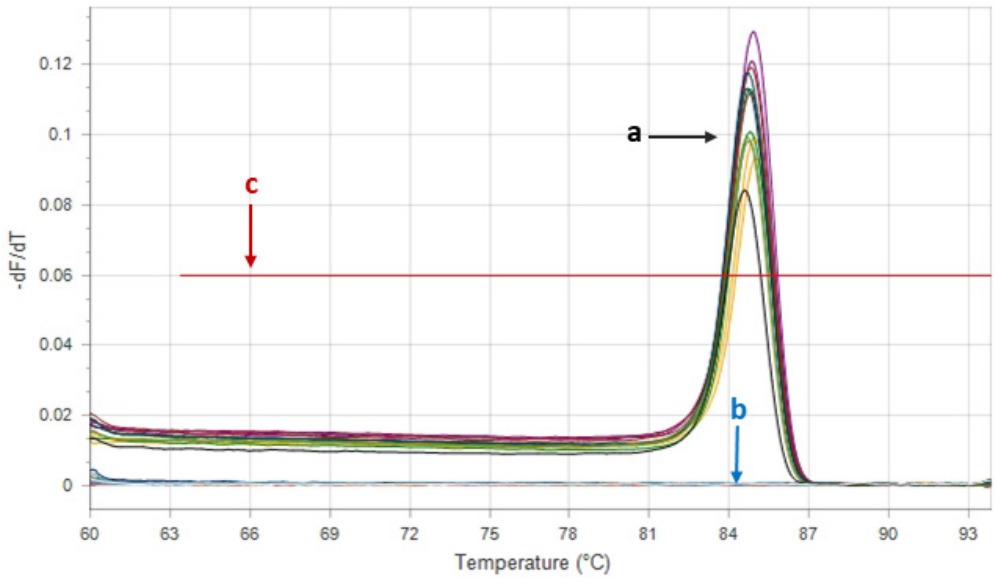

Figure 4.
Melting curve analysis for Brucella spp. in qPCR; a: Positive control and positive unknown samples; b: Negative control and negative unknown samples; c: Threshold line

they were considered positive. All samples were tested twice, and if the qPCR results for both times were positive, that sample was reported positive for the presence of Brucella spp.

Statistical Analysis

Contingency 2 × 2 tables were created to determine the sensitivity, specificity, PV+, PV-, and LRs of positive and negative test results for Rose Bengal and bacterial culture tests, where the result of qPCR was considered standard. The agreement between the tests was evaluated using Cohen's Kappa statistics. According to Landis *et al.* [49], the interpretation of the agreement varied depending on the estimated Kappa values. In detail, when the values were 0-0.20, the agreement was considered slight, but for values above 0.80, it was deemed almost perfect. When the Kappa values were 0.21-0.40, the agreement was considered fair, whereas values of 0.41-0.60 corresponded to a moderate level of agreement. Similarly, a substantial level of agreement was interpreted for values 0.61-0.80. The SPSS software version 16.0 was used for statistical analysis.

Statement of Animal Rights

This study with grant number 3/57600 received ethical approval from the Committee on Research Ethics IR.UM.REC.1401.063, which adheres to the ethical guidelines of research from the School of Veterinary Medicine, Ferdowsi University of Mashhad.

Authors' Contributions

Rahmani.Hk., Hashemi.K., Mirshokraei.P. Conceived and designed the experiments: Aminzadeh. M.J. prepared samples. Aminzadeh. M.J., Khaleghnia.N. Performed the experiment and contributed to preparing reagents, materials and analysis tools. Azizzadeh.M. analyzed data and results. Aminzadeh.M.J and Khaleghnia.N. Wrote the manuscript.

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Competing Interests

The authors declare no conflict of interest.

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